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Convenient synthesis of β-galactosyl nucleosides using the marine β-galactosidase from *Aplysia fasciata*

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Abstract

A convenient synthesis of β-galactosyl derivatives of antiviral and anticancer nucleosides, which utilizes the purified β-galactosidase activity from the hapatopancreas of *Aplysia fasciata*, is reported. All reactions were extremely stereo- and regioselective, since only anomerically pure 5′-*O*-β-galactosyl conjugates were formed. 5′-*O*-β-Galactosyl-5-fluorouridine was synthesized with a 60% yield and 5′-*O*-β-galactosyl-3′-azido-3′-deoxythymidine, the derivative of the anti-HIV drug, was obtained in 43% yield.

Keywords: Enzyme catalysis; Glycosides; Nucleotides; Anticancer agents; Marine organisms

1. Introduction

Nucleoside analogues play an important role in the antiviral and anticancer chemotherapy. It has been found that many natural antibiotics possessing significant antitumor and antiviral activities have the structure of a nucleoside connected to oligosaccharides [1,2]. Several synthetic analogs of naturally occurring nucleosides, such as β-D-arabinofuranosylcytosine (ara-C), 3′-azido-3′-deoxythymidine (AZT) and 5-fluorouracil 1-β-D-ribofuranoside (5-fluorouridine), are clinically useful anticancer or antiviral agents [3,4]. A practical problem with their use arises from the low selectivity of such agents: they kill normal cells, albeit more slowly, as well as neoplastic or virally infected ones. For this reason recent research has focused on the synthesis of new prodrugs, which have lower cytotoxicity than the active substance [5,6]. The prodrug approach represents a very promising method to control the drug concentration in plasma; it involves the chemical modification of a drug into a bioreversible form in order to change its pharmaceutical and pharmacokinetic properties.

Covalent oligonucleotide–carbohydrate conjugates have provided several advantages that benefit cellular uptake, in vivo organ localization and therapeutic efficiency. These advantages are: low toxicity of the components of the conjugates, even at elevated levels; the defined structure and architecture of oligonucleotide–carbohydrate conjugates, which make them easily characterizable by standard biochemical or physical methods respect to polymer conjugates [7]. Glycosylation of the nucleoside can mask the toxicity of the drug until it is unmasked inside a cancer cell by the action of a glycosyl hydrolase. For example, 5′-*O*-β-galactosyl-5-fluorouridine is a prodrug that is more than 100 folds less toxic than the drug to bone marrow cells in Balb/c mice [8]. New glycosyl derivatives of AZT, the potent inhibitor of HIV replication, were also synthesized, and their pharmacokinetic profiles were evaluated [9].

The 5′ primary hydroxyl group of pentofuranoses in nucleosides is surprisingly unreactive [10,11]. It has been shown that a possible cause of this lack of reactivity is due to intramolecular hydrogen bonding between the 5′-hydroxyl group and the nucleoside base [12]. Hence enzymatic methods for the synthesis of nucleoside derivatives seemed to be advantageous over their chemical counterparts in terms of efficiency and stereo- and regioselectivity. Only few reports have appeared in literature in the last 10 years leading with glycosidase-mediated nucleoside glycosylation [13].

In connection with our ongoing project on the search for new glycosidases from marine organisms, we focused our attention on the sea hare *Aplysia fasciata* Poiret 1789, a large mollusc easily collectable and very common in Mediterranean habitats belonging to the order *Anaspidea* [14]. We found a potent β-

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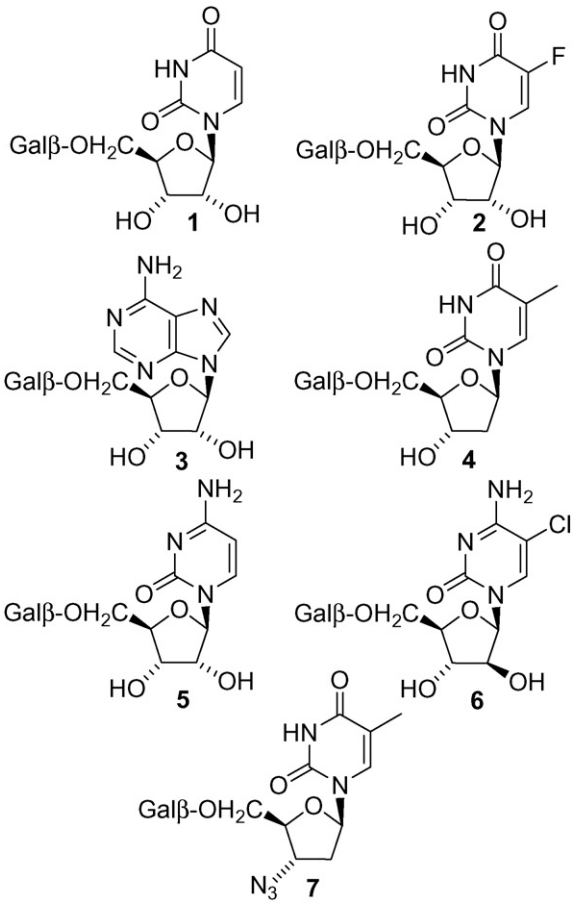

Fig. 1. Structures of the reaction products obtained from galactosylation of nucleotide derivatives.

galactosidase activity in the crude extract from hepatopancreas of this organism, an order of magnitude higher with respect to other mollusc extracts. This crude β-galactosidase activity was utilized for the synthesis of different galactosides [15].

In this paper, we describe a convenient synthesis of β-galactosyl derivatives (Fig. 1) of several nucleoside bases, which utilizes the purified β-galactosidase activity from the hepatopancreas of *A. fasciata*.

2. Experimental

2.1. Materials and methods

SP-Sepharose Fast Flow, DEAE-Sephacel and Superdex-200 were purchased from Amersham Pharmacia Biotech. Nitrophenyl glycosides and nucleosides were obtained from Sigma (St. Louis, MO). Reverse-phase silica gel and TLC silica gel plates were purchased from E. Merck (Darmstadt, Germany). All other chemicals were of analytical grade. NMR studies of native galactosylation products or acetylated derivatives were carried out using Bruker instruments 300 or 400 MHz in D_2O for underivatized compounds and $CDCl_3$ for acetylated products. ESI-MS spectra were obtained on a Q-Tof mass spectrometer, Micro (Micromass). All the chromatographic steps for protein purification were carried at room temperature using an ÄKTA-Prime system (Amersham Pharmacia Biotech). Electrophoresis reagents and equipment were from Bio-Rad. Protein concentrations were routinely estimated using the Bio-Rad Protein System, with the bovine serum albumin as the standard.

2.2. Enzyme source and purification procedure

Ten animals were dissected in order to separate the hepatopancreas from the rest of the visceral mass (including digestive, excretory, blood-vascular, and reproductive systems) and from the external parts. The hepatopancreas (50 g) mass was homogenized in 150 ml of ice-cold 50 mM K-acetate buffer pH 5.5 and a clear protein solution was obtained by centrifugation (33,000 × *g*, 1 h, 5 °C). Solid ammonium sulphate was then added at 4 °C under continuous stirring to give 70% saturation. The precipitated proteins were recovered by centrifugation (33,000 × *g*, 30 min, 5 °C), dissolved in 50 mM K-phosphate buffer pH 6.5 (buffer A) and extensively dialyzed against buffer A. The dialyzed sample was applied to DEAE-Sephacel column equilibrated with buffer A. The column was washed with the equilibration buffer until the absorbance at 280 nm returned to baseline, and retained proteins were eluted with a linear gradient (20-fold column volume) from 0 to 70% 1 M NaCl. The active fractions were pooled and dialyzed in 50 mM Na-acetate pH 3.7 and were loaded onto a SP-Sepharose Fast Flow column equilibrated with the same buffer. After washing the column until the absorbance at 280 nm returned to baseline, retained proteins were eluted with linear gradient (twenty fold column volume) of 0–100% 1 M NaCl. The active fractions judged pure by SDS-PAGE were separately collected; the active fractions containing impurities were loaded again (after dialysis in 50 mM Na-acetate pH 3.7) onto the same column in order to increase the purity. All the pure active fractions were dialyzed in 50 mM K-acetate pH 5.5 and concentrated by ultrafiltration.

2.3. Activity assays

β-D-Galactosidase activity was routinely assayed under standard conditions at 36 °C in 50 mM K-acetate pH 5.5 using 10 mM *p*-nitrophenyl β-D-galactopyranoside (pNP-β-Gal). The reaction was initiated by addition of an appropriate amount of the protein solution to the reaction mixture (0.5 ml). Aliquots (0.05 ml) were withdrawn at time intervals and the reaction was stopped with the addition of Na_2CO_3 1 M (0.45 ml). The increase in absorbance as result of *p*-nitrophenol liberation ($\varepsilon_{405\,nm}$ 18,300 $M^{-1}\,cm^{-1}$) was measured using an Agilent UV–vis Spectroscopy System. One unit of glycosidase activity was defined as that amount of enzyme required to catalyze the release of 1.0 μmol of *p*-nitrophenol per minute. β-D-Galactosidase activity as a function of pH was measured at 36 °C in the presence of 10 mM. pNP-β-Gal at different pH values in different buffers (50 mM K-acetate for pH 5.5–3.7, 50 mM Na-citrate for 3.9–3.4, glycine-HCl for 3.4–2.3). β-D-Galactosidase activity as a function of temperature was measured in the range 20–80 °C, in 50 mM K-acetate pH 3.75, in the presence of 10 mM pNP-β-Gal. Kinetic parameters (K_M, V_{max} and k_{cat}) for pNP-β-Gal and oNP-β-Gal ($\varepsilon_{420\,nm}$ 4500 $M^{-1}\,cm^{-1}$) were determined at 50 °C in

50 mM K-acetate pH 4.5, substrate concentration ranging from 0.5 to 20 mM. Data were fitted with the Michaelis–Menten equation. The pH stability was investigated by incubating the enzyme at 36 °C at different pH values: 50 mM K-acetate pH 4.7, 50 mM Na-citrate buffer pH 3.9, 50 mM glycine-HCl pH 2.8. The pH stability was also tested at 50 °C in 50 mM K-acetate buffer at pH 4.5. Aliquots were withdrawn at intervals and the residual enzymatic activity was measured under standard conditions.

2.4. Estimation of molecular mass

The molecular mass of native β-D-galactosidase was estimated by gel filtration on a Superdex-200 column equilibrated with 50 mM K-acetate pH 5.5, which had previously been calibrated with aldolase (158 kDa), catalase (232 kDa), ferritin (440 kDa) and thyroglobulin (669 kDa). Blue dextran and pyridoxal phosphate were used to determine the void and the total volume, respectively. The flow rate was 1 ml min^{-1}. The molecular mass under denaturing conditions was estimated by SDS-PAGE on slab gel containing 7% acrylamide. Proteins were located on the gels using Comassie Brillant Blue staining.

2.5. General procedure for transglycosylation reaction

Thirty milligram of oNP β-D-galactopyranoside (0.1 mmol) were dissolved in 1 ml of 50 mM K-acetate buffer pH 4.5 with 5 equivalents of nucleoside base (uridine, fluorouridine, thymidine, adenosine, cytidine, 5-chlorocytosine arabinoside, 3′-azido-3′-deoxythymidine). After 4 μg of β-galactosidase were added, the mixture was incubated at 50 °C up to total donor consumption (1–5 h; TLC monitoring EtOAc/MeOH/H_2O 70:20:10, v/v/v). The reactions were stopped by heating the mixtures at 90–100 °C for 2 min. Reaction mixtures were initially purified by reverse phase chromatography RP18 (water/methanol gradients); then products were isolated by silica gel chromatography (eluent: EtOAc/MeOH/H_2O 70:20:10, v/v/v). The products were detected on TLC by α-naphthol reagent and UV absorbance. Acetylation was performed overnight in Ac_2O/pyridine 1:2 at room temperature.

NMR data for products **1**–**5** are referred to acetylated derivatives.

1 ^{1}H NMR (^{13}C NMR): (gal moiety) 4.56 (H-1, d, J = 7.8; 100.8), 5.18 (H-2; 68.5), 5.06 (H-3; 70.2), 5.43 (H-4; 66.9), 3.94 (H-5; 71.0), 4.19–4.12 (H-6; 61.0); (ribose) 6.30 (H-1; 85.5), 5.09 (H-2; 73.2), 5.35 (H-3; 71.2), 4.24 (H-4; 82.0), 4.25–3.69 (H-5; 67.8). ESI MS 681 *m/z* for [M+Na]+. **2** ^{1}H NMR (^{13}C NMR): (gal moiety) 4.56 (H-1, d, J = 7.5; 100.5), 5.24 (H-2; 68.6), 5.07 (H-3; 71.1), 5.42 (H-4; 66.7), 3.94 (H-5; 70.3), 4.21–4.12 (H-6; 61.1); (ribose) 6.31 (H-1; 85.9), 5.06 (H-2; 73.4), 5.36 (H-3; 71.7), 4.27 (H-4; 82.4), 3.70 (H-5; 68.1). ESI MS 699 *m/z* for [M+Na]+. **3** ^{1}H NMR (^{13}C NMR): (gal moiety) 4.60 (H-1, d, J = 7.8; 100.5), 5.24 (H-2; 68.7), 5.07 (H-3; 70.2), 5.43 (H-4;66.7), 3.94 (H-5; 70.9), 4.18–4.12 (H-6; 61.1); (ribose) 6.52 (H-1; 85.0), 5.53 (H-2; 71.7), 5.55 (H-3; 74.0), 4.39 (H-4; 82.6), 4.28–3.75 (H-5; 67.5). ESI MS 682 *m/z* for [M+H]+. **4** ^{1}H NMR (^{13}C NMR): (gal moiety) 4.50 (H-1, d, J = 7.8; 101.1), 5.21 (H-2; 68.3), 5.08 (H-3; 70.1), 5.44 (H-4; 66.7), 3.96 (H-5; 71.0), 4.17 (H-6; 60.7); (2-deoxy ribose) 6.45 (H-1; 84.5), 2.33–2.18 (H-2; 36.7), 5.17 (H-3; 75.3), 4.17 (H-4; 83.2), 4.27–3.75 (H-5; 69.8). ESI MS 679 *m/z* for [M+Na]+. **5** ^{1}H NMR (^{13}C NMR): (gal moiety) 4.56 (H-1, d, J = 7.8; 100.8), 5.12 (H-2; 68.2), 5.06 (H-3; 70.4), 5.45 (H-4;66.8), 3.94 (H-5; 71.2), 4.25–4.14 (H-6; 61.0); (ribose) 6.35 (H-1; 87.5), 5.17 (H-2; 74.5), 5.34 (H-3; 70.8), 4.35 (H-4; 82.8), 4.31–3.70 (H-5; 67.5). ESI MS 680 *m/z* for [M+Na]+. **6** ^{1}H NMR (^{13}C NMR): (gal moiety) 4.35 (H-1, d, J = 7.5; 105.5), 3.63 (H-2; 72.5), 3.52 (H-3; 74.9), 3.88 (H-4; 70.3), 3.57 (H-5; 76.82), 3.80 (H-6; 62.6); (arabinose) 6.19 (H-1; 88.5), 4.24 (H-2; 76.87), 4.20 (H-3; 78.0), 4.11 (H-4; 85.1), 4.22 (H-5; 70.1). ESI MS 462 *m/z* for [M+Na]+. **7** ^{1}H NMR (^{13}C NMR): (gal moiety) 4.33 (H-1, d, J = 7.6; 104.7), 3.60 (H-2; 72.1), 3.51 (H-3; 74.7), 3.88 (H-4; 69.9), 3.57 (H-5; 76.6), 3.79 (H-6; 62.2) (2-deoxy-3-azido ribose) 6.24 (H-1; 86.0), 2.48–2.38 (H-2; 37.9), 4.57 (H-3; 62.5), 4.14 (H-4; 84.4), 4.27–3.78 (H-5; 69.8). ESI MS 452 *m/z* for [M+Na]+.

2.6. Time course reaction

The time course of fluorouridine reaction was carried out in standard conditions using a solution of 0.1 M oNP-β-Gal and 3 equivalents of acceptor. Aliquots were withdrawn at different time intervals and lyophilised after inactivation of the enzyme. The residue was dissolved in D_2O (0.4 ml) with the internal standard (tBuOH) and measured by 400 MHz ^{1}H NMR (256 scans). The ratio of the ^{1}H signals was calculated from the integral curves.

3. Results and discussion

In the preliminary reactions performed by using the crude homogenate from *A. fasciata*, this catalytic activity attracted our attention in that it was able to synthesize β-galactosides in good yields with interesting regioselectivity towards secondary hydroxyl groups [14]. Good results with polar acceptors and the uncommon β-1–3 selectivity in the transgalactosylation reactions with most of the acceptors were observed [15]. These findings prompted us to the purification and characterization of this enzyme.

The purification procedure (see Section 2) allowed to obtain a β-galactosidase in an homogeneous form as suggested by analytical gel filtration on a Superdex-200 column which revealed a single peak and by SDS-PAGE analysis showing a single band. We measured a molecular mass of 164 kDa from gel filtration and a molecular mass of 78 kDa from SDS-PAGE. These data indicate that the purified enzyme is a homodimer.

In order to exploit synthetic properties of the purified β-galactosidase from *A. fasciata*, it was necessary to outline the best operating conditions. β-D-Galactosidase activity measured as a function of pH revealed that the activity increases going from pH 5.5 to 2.3 and drastically drops at lower pHs. When β-D-galactosidase activity was measured as a function of temperature at pH 3.7 it was observed a maximum activity around 75 °C. Stability against irreversible processes was measured at 36 °C at pH 4.7, 3.9 and 2.8. The enzyme completely retained its activity after 23 h incubation at pH 4.7 in K-acetate buffer,

while retained 81% of its activity when incubated at pH 3.9 in Na-citrate buffer for 23 h. At pH 2.8 in glycine-HCl, the half-life time was 0.6 h. Stability was also measured at 50 °C in K-acetate buffer pH 4.5; in this case the enzyme showed a half-life of 15 h. Taking into account these findings pH 4.5 and 50 °C were chosen as standard conditions to perform transglycosylation reactions.

As far as the substrate specificity is concerned we verified that the pure enzyme is highly specific for pNP-β-D-galactopyranoside (pNP-β-Gal), being completely not active on other substrates such as pNP-β-D-glucopyranoside, pNP-β-D-fucopyranoside, pNP-β-D-mannopyranoside, and pNP-β-D-xylopyranoside. The K_M and V_{max} values for pNP-β-Gal at 50 °C in 50 mM K-acetate pH 4.5 were determined to be 1.53 mM and 74.2 U mg^{-1} while for oNP-β-Gal the K_M and V_{max} were 1.47 mM and 101 U mg^{-1}, respectively. Assuming that the dimer is the smallest catalytic unit with a molecular mass of 164 kDa, a k_{cat} of 203 s^{-1} was calculated for pNP-β-Gal and 276 s^{-1} for oNP-β-Gal.

o-Nitrophenyl β-D-galactopyranoside (oNP-β-Gal) was used as glycosyl donor in reactions with 5 equivalents of nucleoside acceptors. When oNP-β-Gal was incubated with β-D-galactosidase from *A. fasciata*, the transfer product was monitored by TLC. After chromatographic purification, ^{1}H NMR spectra, DEPT, COSY and ^{1}H-^{13}C NMR correlation experiments permitted the assignments of chemical shifts of the products and the unambiguous structure assignment. In the COSY spectrum starting from the proton signal of pentose carbon linked to the nucleobase and following the correlations through furanosidic protons it is easy to detect the position of glycosylation for the upfield shift of the signal due to the absence of acetyl group and downfield shift of the corresponding carbon signal due to the galactosylation.

Substrate conversion (hydrolysis plus transgalactosylation) was always greater than 90%. Formation of autocondensation products of oNP-β-Gal was observed only in the reaction of chlorocytidine, for which 8% of oNP-galactosyl disaccharides was isolated. All the products (Fig. 1) were characterized as native or acetylated derivatives.

All reactions were extremely regioselective, since only the 5′-*O*-β-galactosyl product was obtained, while the absolute anomeric purity of the product is a characteristic of glycosidase-mediated synthesis. Reaction yields were calculated respect to the total amount of the donor oNP-β-Gal on the basis of isolated products. The best result was observed in the reaction of uridine, for which an 80% yield of product **1** was obtained. Fluorouridine, which has a very similar structure, except for a fluorine in position 5 of the nucleobase, reacted in a slightly less extent (**2**, 60%). Reactions of adenosine and thymidine afforded the 5′-*O*-β-galactosylated products **3** and **4** in 13 and 12% yields, respectively, which is still a good result, considering that the synthesis conducted with *Aspergillus oryzae* β-galactosidase gave the same products with a yield lower than 5% [16]. Cytidine was galactosylated leading to the product **5** with a yield of 41%. Cytosine β-D-arabinofuranoside (ara-C) was not a substrate for the enzyme, inhibiting the hydrolysis of oNP-β-Gal at concentrations higher than 0.3 M, while 5-chlorocytosine arabinoside (5-Chloro-Ara C) reacted in 12% yield (**6**). These results clearly indicate a preference of the enzyme for ribose in the sugar moiety of the nucleosides. A galactosyl derivative of AZT was chemically synthesized in three steps in 45% yield [9]. A similar result (43%) was obtained by enzymatic galactosylation of AZT conducted using *A. fasciata* β-galactosidase, which afforded product **7** in one step.

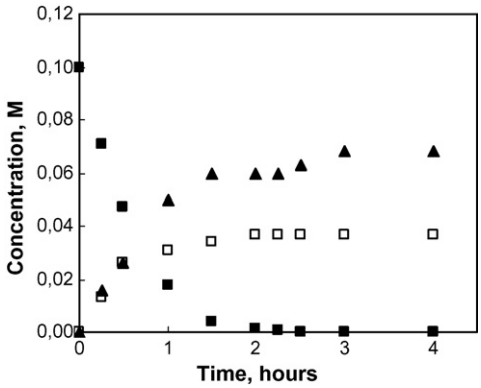

Fig. 2. Time course of reaction with pNP-β-D-Gal and fluorouridine. The curves indicate the concentration of the substrate (pNP-β-D-Gal ■) and products (5′-*O*-β-Gal fluorouridine ▲, D-galactose □) as obtained by integrating peak areas in ^{1}H NMR experiments.

To obtain more detailed information on the synthesis of the prodrug 5′-*O*-β-galactosyl-5-fluorouridine, we monitored the reaction by ^{1}H NMR spectroscopy. The time course of reaction (Fig. 2) indicates that after 2 h the hydrolysis of the substrate is complete; once formation of compound **2** reached its maximum, no decomposition of product could be observed up to 4 h. Reaction composition was still the same even after 24 h, showing that the product **2** is not substrate of the enzyme.

4. Conclusion

In summary, the β-galactosidase found in the visceral mass of *A. fasciata* was isolated and characterized in order to find the best operating conditions in synthesis. This enzymatic activity was utilized for the assembling of several galactosyl derivatives of nucleosides. Enzyme regioselectivity was extremely high, since in all reactions only the product of galactosylation in 5′ position of the nucleoside was observed. Reaction yields were satisfactory in most cases, and very high for uridine derivatives. In particular, 5′-*O*-β-galactosyl-5-fluorouridine, the galactosylated derivative of the anticancer drug fluorouridine, was synthesized with a 60% yield, and 5′-*O*-β-galactosyl-3′-azido-3′-deoxythymidine, the derivative of the anti-HIV drug, was obtained in 43% yield. This is the first report dealing with a glycosyl hydrolase used for modification of nucleosides with convenient yields respect to enzymes from other sources.

Marine environment is far from being well exploited as a source of new enzymatic activities and a thorough knowledge of marine biology and biochemistry together with genetic analysis will be fundamental for this purpose. In the light of recent successes of genetic engineering of marine organisms, nowadays

and future technology will present practical and economically viable alternatives to environmentally destructive large-scale natural collections of marine biomass for enzyme production.

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